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Hyperplastic Lesions of the Primitive Meninx of Fathead Minnows, 'Pimephales promelas' Induced by Ammonia: Species Potential for Carcinogen Testing

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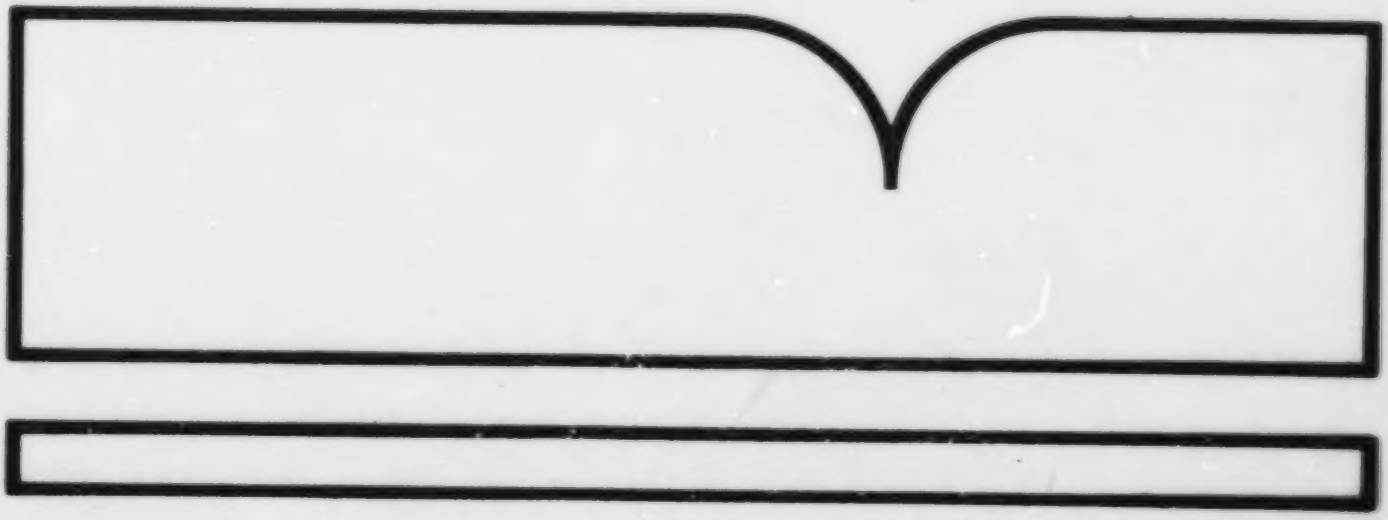

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Hyperplastic Lesions of the Primitive Meninx of Fathead Minnows, *Pimephales promelas*, Induced by Ammonia: Species Potential for Carcinogen Testing[1, 2]

Charlie E. Smith[3, 4]

ABSTRACT—In our laboratory, fathead minnows were reared from time of hatching to adulthood at 5 concentrations of ammonia in a toxicity test lasting 304 days. The ammonia concentration test range was 9.36 to 30.0 mg NH_3-N/liter (0.639 to 2.17 mg NH_3/liter). Intracerebral lesions were observed in fish from each ammonia concentration but not in the controls. The severity of the lesions was positively correlated with increased ammonia concentration. Massive hyperplastic lesions developed in fish tested at the 2 highest concentrations. Histologically, lesions appeared to originate from the primitive meninx, which grew into the cranial cavity, and in some fish through the cranium. Based on the orderly appearance of tissues and evidence of regression of lesions, the lesions are not considered to be neoplasms but hyperplasias of primitive meninx tissue induced as a response to ammonia exposure. Fathead minnows (*Pimephales promelas*) have proved useful for acute toxicity testing of environmental contaminants and have a potential usefulness in carcinogenicity testing. They are easily maintained in the laboratory, can be readily induced to spawn during their first year of life, and have a relatively short period of embryogenesis. — Natl Cancer Inst Monogr 65: 119–125, 1984.

GEOGRAPHICAL RANGE

The fathead minnow (*Pimephales promelas*, family Cyprinidae) is probably the most easily propagated minnow indigenous to a geographical range including southern Canada, the United States, and northern Mexico. Viable populations are present from sea level to an elevation of 3,000 m (*1*). The species is widely cultured for bait and forage fish and is used extensively at various laboratories throughout the United States (*2*) as an assay fish for the determination of toxicity of environmental pollutants.

ABBREVIATIONS: DMBA = 7,12-dimethylbenz[*a*]anthracene; H & E = hematoxylin and eosin.

[1] Presented at the Symposium on the Use of Small Fish Species in Carcinogenicity Testing, National Institutes of Health, Bethesda, Maryland, December 8–10, 1981.

[2] Supported in part by grants R803950 and CR807240 from the Environmental Research Laboratory of the United States Environmental Protection Agency, Duluth, Minnesota.

[3] Fish Cultural Development Center, United States Fish and Wildlife Service, Bozeman, Montana 59715.

[4] I thank Mr. John Morrison, Ms. Elizabeth Meyn, and Dr. Robert V. Thurston for their technical assistance.

APPEARANCE

Fathead minnows are typical of cyprinids in appearance and average 50–70 mm in length, with a maximum length of about 100 mm. Prior to spawning, fish exhibit sexual dimorphism; the male develops a generally black color with vertical bands of golden brown, a swollen blunt head with breeding tubercles (pearl organs), and a thickened rugose nape pad resulting from increases in epidermal and dermal thickness and mucous cell number (*3*), as illustrated in figure 1. The female is silvery to olivaceous in color, has a more pointed head than the male, and, when gravid, has a definite distended abdomen. The surest and easiest way one can recognize sexes is the presence of an ovipositor in the female (fig. 2); this can usually be seen by flexing the fish and thereby putting pressure on the abdomen (*4*).

LIFE CYCLE

When exposed to seasonal environmental conditions, the fathead minnow is an intermittent, multiple-spawning fish. Spawning occurs during the summer, generally within the range of 18° to 30° C. Before spawning, the male locates and defends a suitable nesting site beneath floating objects or vegetation; in the laboratory, spawning will occur on the undersurface of spawning tiles. During spawning, eggs are attached to the underside of the nest by an adhesive coating on the chorion. After eggs are deposited and fertilized, the female is driven from the nest by the male (*2*). Embryogenesis takes approximately 5 to 6 days at 25° C. Eggs are about 1.0–1.25 mm in diameter and are laid in clusters, usually 1 egg deep in groups of about 250 to 500. More than 1 female may deposit eggs on the same nest cared for by a single male, and as many as 12,000 eggs/nest have been recorded (*5*).

Both temperature and photoperiod control spawning by fathead minnows. Various minimum temperatures and dates of first spawning have been observed by different investigators, and, although some minimum temperature requirement is indicated, the large number of first spawnings in May strongly support day length as the major influence initiating spawning activity. However, temperature may be more important than photoperiod once the reproductive cycle has been started. Actual first date of spawning is probably determined by complex interrelationships involving temperature, light, and severity of the preceding winter with its effects on the general condition of the fish as spawning approaches and gonads mature (*2*).

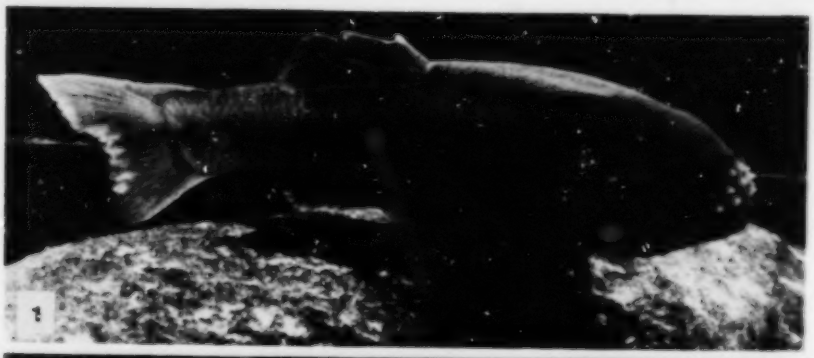

FIGURE 1.—Adult male fathead minnow. *Note* breeding tubercles on snout and thickened pad anterior to dorsal fin. (Figure is used through the courtesy of S. Flickinger.)

FIGURE 2.—Adult female fathead minnow. *Note* swollen abdomen and the presence of an ovipositor. (Figure is used through the courtesy of S. Flickinger.)

A mature female may be expected to produce between 1,000 and 10,000 eggs from several spawnings over the course of a breeding season, with the total number depending primarily on female size and environmental conditions (*2*).

Under controlled spawning in the laboratory environment, fathead minnow eggs may be obtained throughout the year. This is accomplished with maintenance of optimum temperature and photoperiod (*6*). Till (*7*) described a laboratory technique for obtaining freshly fertilized eggs for use in toxicity experiments. Basic embryological information on the fathead minnow describes development of the embryo from fertilization through the 13-somite stage (*8*) as shown in table 1.

TABLE 1.—*Fathead minnow stages*[a]

Stage No.	Stage name	Time from fertilization, hr[b]
1	Fertilization	0.0
2	2-Cell	1.50
3	4-Cell	2.00
4	8-Cell	2.50
5	16-Cell	2.75
6	Late cleavage	4.00
7	Blastula	5.00
8	Gastrula	7.00–17.00
9	Neurula	16.00
10	Head formation	18.00
11	5-Somite	24.00
12	13-Somite	33.00

[a] Data are reproduced with permission from (*8*).
[b] Temperature at fertilization was 23° C.

REARING REQUIREMENTS

Space may be a limiting factor for the number of fish which can be supported within a contained area. Optimum stocking density of fathead minnow fry in ponds is 100,000–300,000 fish/acre (at 1/4 hectare) or about 0.05 fish/liter (*2, 4*). Smith et al. (*9*) established a tolerance density of 0.17 fish/liter in small 90-liter laboratory tanks. Generally in most chronic toxicity tests, 40–50 fry are placed in 60- to 90-liter aquaria. This number is usually reduced to 15 fish at the end of 60 days and further reduced 6–8 months later at the onset of the spawning period.

In his study on oxygen requirements of fathead minnows, Brungs (*10*) reported that the number of eggs per female was reduced at 2.0 mg/liter, and no spawning occurred at 1.0 mg/liter. Furthermore, fry survival was reduced at 4.0 mg/liter, and approximately one-fifth of those fry that did survive at that concentration were deformed. Recommended oxygen concentrations for fathead minnow toxicity tests is 60% of saturation or greater (*6*). Water temperature and pH have been shown to have a definite influence on reproduction of fathead minnows. Brungs (*11*) exposed fathead minnows continuously to elevated water temperatures ranging from 26° to 34° C. He found that reproduction was more sensitive than was survival, growth, or egg hatchability in assessing the effect of temperature. Growth was reduced and no spawning or mortality occurred at 32° C.

Mount (*12*) maintained fathead minnows continuously for 13 months in water at different pH values from 4.5 to 7.5. At pH 5.2, females became gravid and sexually mature but did not spawn. Egg production and hatchability were reduced at pH 5.9 and lower, and all eggs were abnormal. A

pH of 6.6 was marginal for vital functions but safe for continuous exposure.

Standard procedures for conducting bioassays with fathead minnows have been established by the United States Environmental Protection Agency (*6*), and procedures for acquisition and culture of research fish are also available (*13*). Fathead minnows adapt readily to fish feeds prepared commercially. Because of their small size, the feed for newly hatched fry must be extremely finely ground meal. In our tests, fish are fed live cultured brine shrimp until they are 15–20 days old at which time a commercial trout fry feed (Silver Cup Salmon) is pulverized and fed with the brine shrimp. When large enough (about 45 days), fish are fed dry feed twice each day with a supplement of live brine shrimp once each week.

PAST STUDIES

Although many reports are available that describe the acute, partially chronic, and chronic toxicity to fathead minnows of herbicides, pesticides, heavy metals, and various organic compounds, we found no published reports involving carcinogenicity testing to toxicants for this species. In vitro fish cell lines developed by Wolf and Quimby (*14*) have been used by some investigators to study the effect of carcinogens in vitro. Bourne and Jones (*15*) studied the effect of DMBA on RTG-2 cells and epithelioid cells of the fathead minnow. Mitotic indexes demonstrated that the RTG-2 fibroblastic cells were distinctly more sensitive to DMBA than were the epithelioid but did not show the multinucleation shown by the latter cells. These authors concluded that the action of DMBA on both cell lines confirmed that they are non-neoplastic.

Thornton et al. (*16*) studied the in vitro metabolism of benzo[*a*]pyrene by bluegill fry-2 and fathead minnow cell lines. Their results show that cells of both lines metabolize a high proportion of this polycyclic hydrocarbon to the 7,8,-diol-glucuronide and thus form a water-soluble conjugate of a proximate carcinogenic metabolite of benzo[*a*]pyrene, which could be transmitted to various tissues within the fish or throughout the aquatic environment.

ADVANTAGES AND SPECIAL PROBLEMS RELATED TO FATHEAD MINNOWS

The fathead minnow occupies a broad natural range from the prairie provinces of Canada and northern United States between the Rockies and Appalachians south to Tennessee, Arkansas, and northern Mexico (*17*). Stocks of fathead minnows are also available from commercial bait farms. Fish used in our studies were captured from a farm pond in eastern Montana.

Although fathead minnows can withstand considerable physical stress in the wild, they sometimes suffer diseases associated with the bacterium *Aeromonas liquefaciens*, the digenetic trematode *Gyrodactylus* sp., and the parasitic copepod *Lernea*. From diseased fish in the laboratory, we have identified the protozoans, *Costia* sp. and *Chilodenella* sp., more often the latter. We have also isolated *A. liquefaciens* and *Pseudomonas fluorescens* from diseased fish. Outbreaks of disease are usually associated with elevated temperatures or crowded conditions, or both. Brungs (*11*) found increased incidence of disease among fathead minnows reared at elevated temperatures.

In wild populations, most males and up to 90% of the females may die after spawning at 2 years; a few fish survive the third year (*4*). Under laboratory conditions, however, broodfish spawn during their first year of life and experience little postspawning mortality.

HYPERPLASTIC LESIONS OF THE PRIMITIVE MENINX INDUCED BY AMMONIA

A test, conducted by investigators at the Fisheries Bioassay Laboratory, Montana State University, Bozeman, determined the effect on fathead minnows of chronic exposure to ammonia. Cultural procedures are similar to those described by the Environmental Protection Agency (*6*).

Fish were exposed for 11 months to 5 concentrations of ammonia within the range 0.639–2.17 mg NH_3/liter, i.e., 9.36–30.0 mg NH_3-N/liter, in 1 control and 5 test tanks (table 2). The temperature was approximately 25° C,

TABLE 2.—*Mean (and range) of water chemistry variables to which fathead minnows were exposed for 11 mo*

Tank No.	Temperature, degrees C	O_2, mg/liter	pH	Total NH_3-N, mg/liter	Un-ionized NH_3, mg/liter
1	25.6 (23.5–26.5)	6.7 (5.6–8.0)	8.08 (7.91–8.30)	0.07 (0.00–0.26)	0.006 (0.000–0.036)
2	25.1 (23.3–25.9)	6.6 (5.4–7.8)	8.02 (7.87–8.29)	9.30 (5.94–10.6)	0.639 (0.427–1.14)
3	25.0 (23.2–25.9)	6.6 (5.2–8.0)	7.98 (7.84–8.20)	12.2 (7.28–13.8)	0.815 (0.508–1.21)
4	25.2 (23.5–26.1)	6.6 (5.6–7.9)	7.97 (7.82–8.18)	16.4 (11.1–18.5)	1.07 (0.756–1.57)
5	25.0 (23.6–25.9)	6.7 (5.7–7.8)	7.96 (7.82–8.16)	19.7 (10.4–22.4)	1.25 (0.715–1.94)
6[a]	24.5 (23.1–25.7)	7.1 (6.2–7.9)	8.05 (7.84–8.16)	30.0 (27.7–32.6)	2.17 (1.36–2.99)

[a] Test in this tank was terminated after 133 days due to excessive mortality of fish.

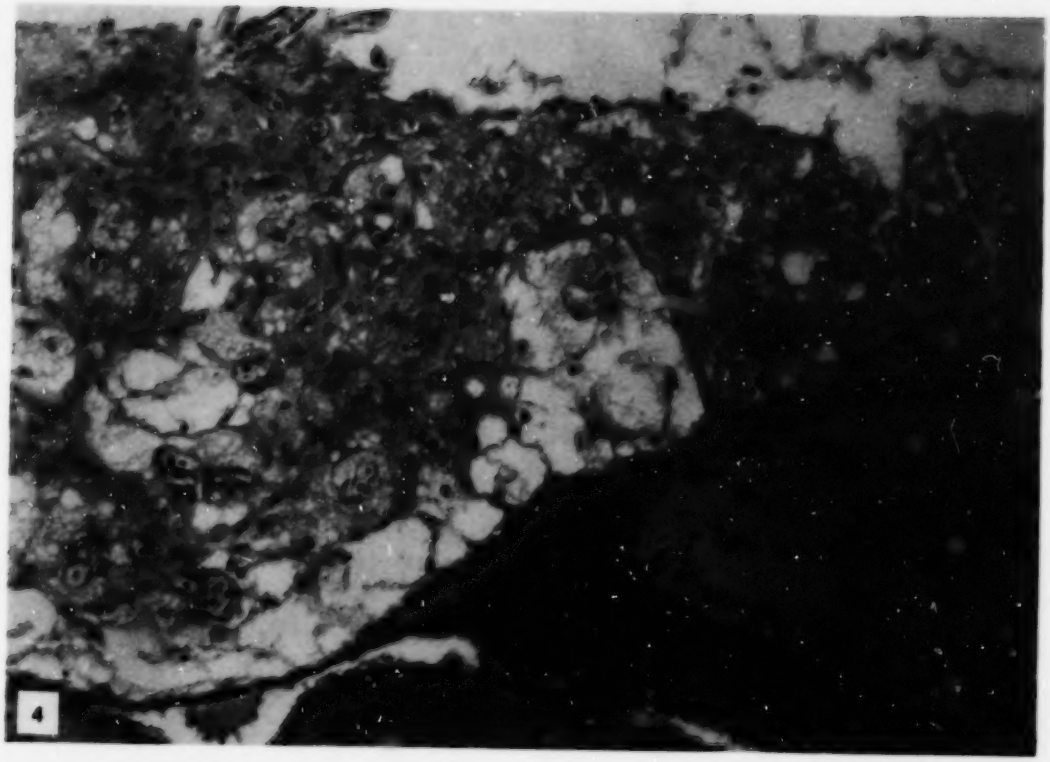

FIGURE 3.—Adult fathead minnow exposed to 1.25 mg NH_3/liter for 150 days showing massive growth on head. Eye has been misplaced ventrolaterally.

FIGURE 4.—Proliferation of cells from primitive meninx of fathead minnow exposed to 1.25 mg NH_3/liter for 60 days. H & E. Bar = 30 μm. × 400

oxygen concentration was 6.7 mg/liter, and the pH was 8.0 in all tanks. Ammonia in the form of ammonium chloride and dilution water was added to the tanks from a proportional diluter of the basic design of Mount and Brungs (*18*). The water exchange rate in each tank was approximately once every 2 hours. At the start of the test, fry between 1 and 5 days old were selected at random from a single pool and 50 were placed in each tank. At 60 days into the test, the number of fish in each tank was reduced to 15, except at the highest concentration on which fewer fish had survived. During the spawning period, the number of sexually mature males in each tank was reduced to no more than 4.

During the course of the study, growths were observed on the heads of several fish held at the 2 highest ammonia concentrations. Swollen darkened areas were noted on the heads of several fish held at lower concentrations. At 60 days when the fish were reduced in number, 7–15 fish were collected for histopathological examination from all but the highest concentration, and 2–8 fish were selected at the end of the study. Periodically, 8 additional fish exhibiting gross lesions were collected for histopathological

examination. All fish were preserved in Bouin's fixative. Paraffin sections were cut at 5 μm and stained with H & E. Some sections were stained with Masson's trichrome, periodic acid-Schiff, and Alcian Blue. Frozen sections were cut from fresh and formalin-fixed material and stained with oil red O.

Grossly and histologically, the severity of lesions was positively correlated with ammonia concentration. One fish exposed to 1.25 mg NH_3/liter developed a lesion sufficiently massive to cause misalignment of the eyes (fig. 3). Histologically, the lesions appeared to be of a cell type that originated from the primitive meninx covering the brain (fig. 4). Hyperplastic tissue often completely surrounded the brain but was not observed around the spinal cord (figs. 5, 6). The cell type varied; many cells were swollen and contained small nuclei and a dense foamy cytoplasm. Rodlet cells were sometimes diffusely scattered throughout and often were concentrated at the periphery (fig. 7). Blood vessels were present throughout the hyperplastic tissue, and many had accumulations of melanin around their walls, thus giving a darkened appearance to the top of the head. Tissue was sometimes dense, especially in areas where it appeared to proliferate from the primitive meninx. Cells resembling adipocytes were scattered throughout. The hyperplastic tissue stained negatively for collagen, mucin, and mucopolysaccharides. An occasional cell stained positive for lipid, but most were negative. Macrophages containing degenerate, periodic acid-Schiff-positive debris were sometimes scattered throughout the tissue, and occasionally focal accumulations of macrophages were seen, especially in advanced lesions in which dystrophic calcification was also apparent.

Of the fish which exhibited gross lesions, 2 were transferred to a freshwater holding tank and maintained for approximately 1 month. During this time, the lesions noticeably regressed. Judged on the orderly appearance of tissues from lesions and evidence of their regression, the lesions observed are not considered to be neoplasms but connective tissue hyperplasia induced as a response to exposure to ammonia.

In summary, fathead minnows are easily maintained within the laboratory, can be induced to spawn their first year of life, have a short period of embryogenesis, and provide adequate numbers of eggs per spawning for testing. This species has proved to be extremely useful in toxicity testing of environmental contaminants and appears to have a potential usefulness in carcinogenicity testing.

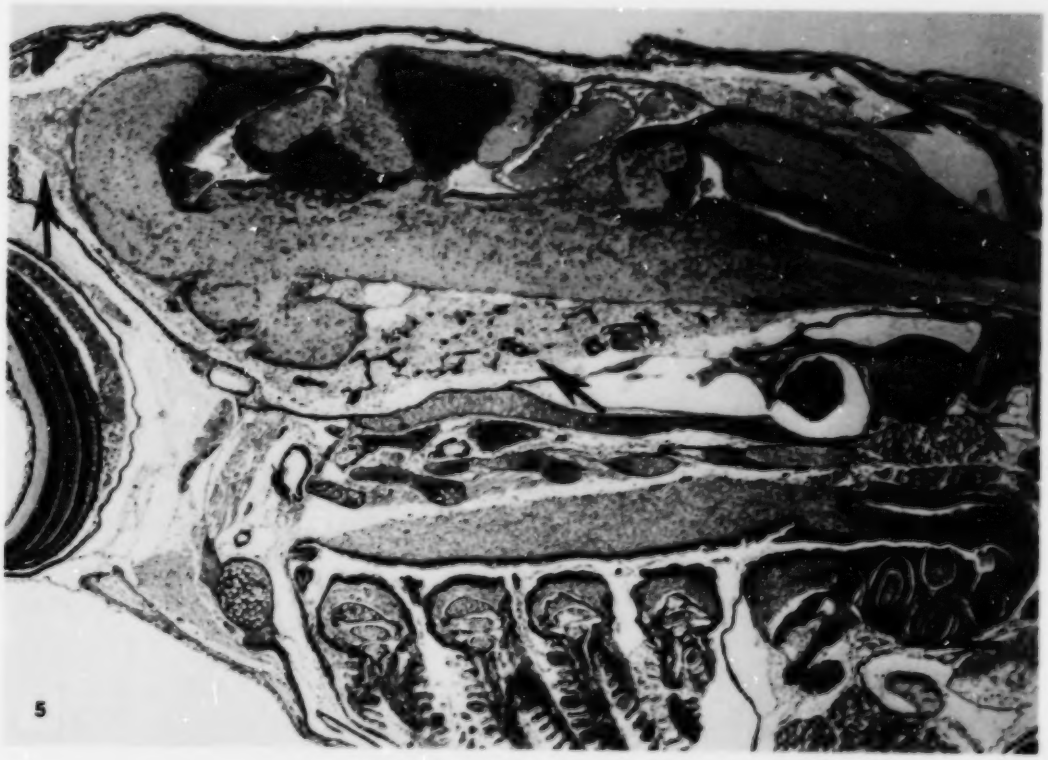

FIGURE 5.—Longitudinal section through brain of fathead minnow exposed to 1.25 mg NH_3/liter for 60 days. *Note* proliferation of tissue in different areas around the brain (*arrows*). H & E. Bar = 100 μm. × 60

FIGURE 6.—Cross section through medulla of a fathead minnow exposed to 1.25 mg NH_3/liter for 60 days. *Note* extensive proliferation of tissue surrounding medulla and the accumulation of melanin around vessels scattered throughout proliferated tissue. H & E. Bar = 100 μm. × 60

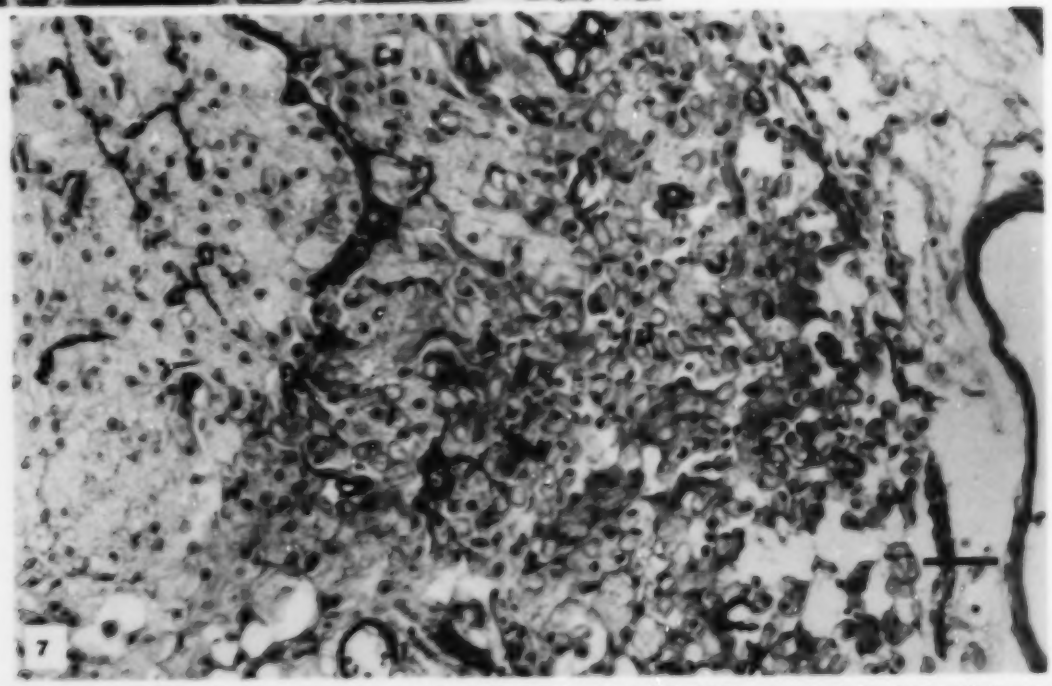

FIGURE 7.—Proliferated tissue containing swollen cells with small nuclei and foamy cytoplasm. Accumulations of rodlet cells are seen at the periphery of foamy cells in *right half* of photomicrograph. H & E. Bar = 30 μm. × 400

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